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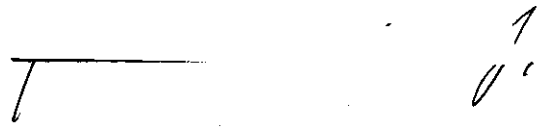

MECHANISM OF THE HYDROLYSIS OF PHOSPHATE MONOESTERS

A THESIS

Presented to

The Faculty of the Graduate Division

by

Monso Pitman Tatum, Jr.

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MECHANISM OF THE HYDROLYSIS OF

PHOSPHATE MONOESTERS

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PREFACE

In recent years intensive interest has been shown in the reactions and reaction mechanisms of nucleophilic substitution on various derivatives of phosphoric acid. The hydrolysis reactions of the monoesters of phosphoric acid are of both theoretical and practical interest, especially in the area of Biological Reactions. Hopefully, the elucidation of the mechanisms of solvolysis of such model compounds will aid in understanding the pathways by which these compounds react in biological systems.

I would like to express my appreciation and thanks to Dr. James R. Cox, Jr. for suggesting this problem and to Dr. John R. Dyer and Dr. Raymond D. Kimbrough, Jr. for reading this thesis. I would also like to thank the National Institutes of Health for their generous financial support.

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CHAPTER I

INTRODUCTION

Early investigations of the hydrolysis reactions of the organic monoesters of orthophosphoric acid found that the hydrolysis rates of the compounds exhibit a distinct dependence upon the acidity of the solvent. More detailed studies by Bailly (1) and later by Desjobert (2) revealed that the rate of hydrolysis of monomethyl phosphate reaches a maximum at pH 4 and decreases as the acidity of the solution is either increased or decreased. In strongly acidic solution the rate again increases while in basic solution the rate reaches a very slow, limiting value. This behavior is illustrated by Figure 1.

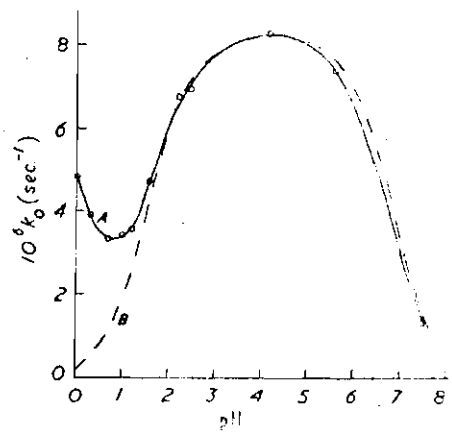

Figure 1. pH Rate Profile

A. Experimental B. Calculated

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1. O. Bailly, Bull. Soc. chim. France, (5), 9, 340 (1942).
2. A. Desjobert, Compt. rend., 224, 575 (1947).

The precise determination of the ionization constants of monomethyl phosphate by Bunton, Llewellyn, Oldham, and Vernon (3) showed that the monoanion, $ROPO_3^-$, is the only species present in significant quantity at pH 4. At higher pH the monoanion loses a proton to form the unreactive dianion, while at high acid concentrations the monoanion gains first one and then two protons to form the neutral ester and its conjugate acid, respectively, both of which undergo solvolysis.

Bunton and co-workers further demonstrated that most of the pH rate profile could be calculated from the rate constant for the reaction at the pH 4 rate maximum and the concentration of the monoanion, as calculated from the ionization constants of the monomethyl phosphate. This correlation, however, failed in the region of high acidity due to acid catalysis of the reaction in that region.

That the monoanion of a monoester of phosphoric acid has a special mechanism available to it for nucleophilic substitution on phosphorus was further demonstrated by a comparison of the rate of the hydrolysis of the monoanion of monomethyl phosphate (4) with that of dimethyl phosphate (5). After suitable corrections were made for the position of bond cleavage it was found that the first-order rate constant for the hydrolysis of the monoanion of monomethyl phosphate at pH 4 is of the order of 100 times that of the second-order rate constant for the reaction of hydroxide ion with dimethyl phosphate monoanion. The nucleophile in the

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3. C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, J. Chem. Soc., 3574 (1958).

4. J. Kumamoto, J. R. Cox, Jr. and F. H. Westheimer, J. Am. Chem. Soc., 78, 4858 (1956).

5. P. C. Haake and F. H. Westheimer, J. Am. Chem. Soc., 83, 248 (1951).

first instance is water, while that in the second instance is hydroxide ion. Even if it were assumed that the hydrolysis of the monoanion of monomethyl phosphate is also first-order in water (an assumption for which there is no experimental justification) the difference in the rates is still of such order of magnitude as to suggest that a different mechanism is operating in the pH 4 region.

Butcher and Westheimer (6) determined that when the phosphate monoester of (-)-1-methoxy-2-propanol is hydrolyzed at pH 4 the alcohol residue is not racemized but retains its configuration, indicating that it is the phosphorus-oxygen bond rather than the carbon-oxygen bond that is cleaved.

Bunton and co-workers (3) found in addition that during the hydrolysis of monomethyl phosphate at pH 4 in water enriched in O^{18} there is no O^{18} enrichment of the alcohol formed. But one atom of O^{18} is found in the inorganic phosphate produced during the reaction. Additional studies of the O^{18} exchange of orthophosphoric acid at pH 4 (7) revealed that the rate of exchange of O^{18} by the monoanion of phosphoric acid with water is one-tenth that of the rate of appearance of inorganic phosphate enriched in O^{18} during the hydrolysis of the monoanion of monomethyl phosphate. Near pH 1 the kinetically significant species is the neutral acid. Solvolysis of this species results in carbon-oxygen bond cleavage. In the regions of higher acidity (one molar perchloric acid and greater) the conjugate acid of the ester undergoes hydrolysis both by carbon-

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6. W. W. Butcher and F. H. Westheimer, J. Am. Chem. Soc., 77, 2420 (1955).

7. C. A. Bunton, D. R. Llewellyn, C. A. Vernon, and V. A. Welch, J. Chem. Soc., 1636 (1961).

oxygen and phosphorus-oxygen bond cleavage, although experimental difficulties make the numbers obtained subject to rather large error.

In all cases that have been carefully investigated in which a clear-cut rate maximum occurs, hydrolysis in the pH region of maximum rate results in exclusive phosphorus-oxygen bond cleavage. It is important to note that this result is valid only in the region of maximum rate, and not for the reactions of the neutral species and of the conjugate acid.

The solvolysis rates are little affected by changes in ionic strength of the solvent, nor is there evidence of general buffer-catalysis (8). Moreover, there is no change in rate when the solvent is changed to deuterium oxide. The rates are also little affected by the nature of the R group, but the rate is slightly increased by making the leaving group the conjugate base of a stronger acid.

It has been difficult to specify a precise pathway for the special mechanism for the hydrolysis of phosphate monoester monoanions as there has been adduced no precise criterion of molecularity. But certain features of the mechanism can be deduced from the available data. Firstly, the reaction must be limited to a species containing an ionized and an un-ionized acidic group. Secondly, as the reaction is not very sensitive to the nature of the R group even though there is some correlation between the stability of the anion, RO^-, and hydrolysis rate, the rate-determining step probably does not involve the separation of the anion. Thirdly, since the reaction is not affected by large changes in the ionic strength of the medium, the transition state does not have a separation of charge.

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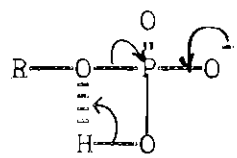
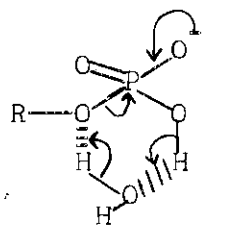
8. C. A. Vernon, Special Publications of the Chemical Society, 8, 17 (1957).

Three mechanisms which are consistent with the above data have been proposed. A fragmentation reaction, during which a proton migrates to the ester oxygen, could afford as immediate products the alcohol and the hypothetical metaphosphate ion (Equation 1) (3). The metaphosphate ion postulated as the intermediate would then undergo rapid hydration by water to orthophosphoric acid. A bimolecular attack of solvent on phosphorus (8) with simultaneous migration of hydrogen to the ester oxygen would also be acceptable in the absence of a criterion for the molecularity of the reaction with respect to the nucleophile.

A third mechanism proposed by Kumamoto and Westheimer requires an intermediate to be formed by hydrogen-bonding a molecule of water to the monoanion. Cyclic decomposition of this intermediate effects proton transfer (9) (Equation 2).



$$\longrightarrow \quad ROH + PO_3^- \qquad (1)$$



$$\longrightarrow \quad ROH + \left[O=P(-O^-)-O \right]^- + H_2O \qquad (2)$$

No enrichment of O^{18} was found in unreacted monomethyl phosphate recovered from the reaction at pH 4 in H_2O^{18} (3), therefore, a mechanism in which water adds to the phosphoryl group followed by the elimination of the elements of alcohol and orthophosphoric acid can be ruled out.

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9. J. Kumamoto and F. H. Westheimer, J. Am. Chem. Soc., 77, 2515 (1955).

Method of Procedure

If indeed one of the proposed mechanisms which require the formation of metaphosphate ion as an intermediate is operative in the hydrolysis of phosphate monoester monoanions experiments can be designed to trap this intermediate.

Often, the nucleophilicity of a reagent is too narrowly considered to be an inherent property of that particular reagent. The relative order of reactivity of different nucleophiles is frequently fairly constant with each of a variety of substrates, but there exist specific interactions between substrate and nucleophile which alter the reactivity ratio of two nucleophiles with one substrate from that with another substrate by an amount which is readily measurable.

This particular relationship is here used to test the mechanism postulated by Butcher and Westheimer (9). If the mechanism has as its slowest step the rate-determining dissociation of the monoanion into alcohol and metaphosphate ion, then two nucleophiles, competing to react with the metaphosphate ion in the product-determining step, will always yield a constant ratio of the two possible products, regardless of the phosphate ester hydrolyzed.

If the reaction involves nucleophilic attack of solvent upon the phosphorus atom in a step which is both rate- and product-determining, it will be expected that the ratio of the two possible products will vary when different phosphate esters are hydrolyzed. Koshland and Herr (10) have investigated the hydrolysis of phosphate esters in solutions containing 12.1 molar methyl alcohol and found that the reactivity ratio of water

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10. D. E. Koshland, Jr. and E. B. Herr, Jr., J. Biol. Chem., 228, 1021 (1957).

and methyl alcohol does vary. On this basis they reasoned that the mechanism postulated by Kumamoto and Westheimer is not operative. However, these studies were carried out in highly acidic solutions in which the neutral species, $ROPO_3H_2$, is in greatest concentration and a mechanism involving nucleophilic attack of solvent accounts for most of the rate. Thus Koshland and Herr's experiments constitute no test of the special mechanism.

From the data taken at pH 4 in the study by Koshland and Herr it appears that methyl alcohol has a reactivity which was large enough to compete with water in the reaction. For this reason methyl alcohol was chosen as a possible competitor with water in the reaction.

Two phosphate esters, para-nitrophenyl and phenyl phosphate, were chosen for use in the present studies because the reactivity of these is significantly different, and they are sufficiently reactive for convenient study. The rate constant for hydrolysis of para-nitrophenyl phosphate is 1.28×10^{-5} sec.$^{-1}$ and that of phenyl phosphate, 2.78×10^{-6} sec.$^{-1}$ at pH 4 at 60^0 C. That of the product, methyl phosphate, is 1.91×10^{-7} sec.$^{-1}$, so that it is essentially unreactive under the reaction conditions during the period required for the reaction of phenyl esters. Also the presence of methyl phosphate does not alter the determination of inorganic phosphate.

CHAPTER II

EXPERIMENTAL

para-Nitrophenyl Phosphate

The sodium salt of para-nitrophenyl phosphate was prepared by the method of Bessey and Love (1). Keeping the temperature at 0° C. 27 grams of para-nitrophenol dissolved in 100 ml. of dry pyridine, was added to 30 ml. of $POCl_3$ dissolved in 200 ml. of dry pyridine in a 1000 ml. three-neck flask. The reaction was stirred in an ice bath for thirty minutes and then poured into 400 ml. of cold water. The mixture was then brought to pH 8 by adding solid NaOH and the solvent stripped with a rotary evaporator. The residue obtained was extracted with boiling 87 per cent ethyl alcohol and washed with cold absolute ethyl alcohol.

The purity of the product was determined by hydrolyzing a 20 milligram sample of the purified product in one molar hydrochloric acid at 100° C. Based on the para-nitrophenol formed upon hydrolysis, the sample was found to be 99 per cent para-nitrophenyl phosphate. The yield of the product was 37 per cent.

Phenyl Phosphate

A method similar to that of Freeman and Clover (2) was used to prepare phenyl phosphate. Nineteen grams of phenol was boiled under

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1. O. A. Bessey and R. H. Love, J. Biol. Chem., 196, 175 (1952).
2. H. F. Freeman and W. C. Clover, J. Am. Chem. Soc., 60, 750 (1938).

reflux with 90 ml. of $POCl_3$ for ten hours and phenylphosphoryl chloride was isolated by fractional distillation. The distillate was dissolved in chloroform and treated with an equivalent amount of water. The crystals thus obtained were recrystallized from chloroform and dried under vacuum. A yield of 28 per cent phenyl phosphate was obtained. Hydrolysis of a sample of the phenyl phosphate gave 98.9 per cent of the calculated yield of inorganic phosphate.

Analytical Techniques

Inorganic phosphate was determined by a modification of the method of Lowry and Lopez (3). Samples, containing known amounts of potassium dihydrogen phosphate, were prepared in 100 ml. volumetric flasks with a 0.05 molar (pH 4) buffer solution of potassium bipthalate. Samples of these solutions were placed in 100 ml. volumetric flasks and five milliliters each of a one per cent ascorbic acid solution and a one per cent solution of ammonium molybdate in 0.05 normal sulfuric acid were added. The flasks were diluted to the mark and read at 700 mμ on a Zeiss spectrophotometer. A plot of absorbance vs. concentration of the known samples was found to be linear between 1 and 7.5×10^{-5} molar phosphate. The readings were reproducible to within less than one per cent error, provided the ascorbic acid and ammonium molybdate solutions had been stored in the cold and excess quantities of the reagents were not used.

Ten milliliter samples from the kinetic runs were diluted in 100 ml. volumetric flasks in order that the concentration of inorganic phosphate be roughly within the limits where the determination was found to be linear.

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3. O. H. Lowry and J. A. Lopez, J. Biol. Chem., 162, 421 (1946).

The extinction coefficient of the para-nitrophenylate ion was found to be 17,500 at 400 mμ as reported by Bessey and Love (1). para-Nitrophenol produced in the reaction was determined by adding 10 ml. of the sample to a volumetric flask and diluting to the mark with six normal sodium hydroxide.

A reliable extinction coefficient for phenolate ion could not be obtained from the literature. It was determined experimentally by measuring the absorbance of a sample of triphenyl phosphate in 0.01 normal base at complete reaction at 291.5 mμ , the λ max. (4). The extinction coefficient was found to be 2400. This value was checked by hydrolysis of a sample of phenyl phosphate in one molar hydrochloric acid at 100° C. Since the inorganic phosphate and the phenol are produced in a one to one ratio, the concentration of phenol could be calculated. From the concentration of phenol and the observed absorbance the extinction coefficient of the phenolate ion was calculated to be 2427. Additional studies indicated that there was no change in the extinction coefficient for the phenolate ion when the concentration of sodium hydroxide was varied from 0.01 to 0.25 molar.

The concentration of phenol from the hydrolysis of phenyl phosphate was determined by adding 10 ml. of the sample to a volumetric flask. To this was added 10 ml. of one molar sodium hydroxide giving a solution approximately 0.1 molar in sodium hydroxide when diluted to the mark.

Kinetic Runs

Kinetic runs of the para-nitrophenyl phosphate were made in

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4. John H. Dixon, Jr., These laboratories, 1963.

solutions containing 4×10^{-3} molar of phosphate ester and buffered at pH 4 with 0.05 molar potassium biphthalate. Due to the absorbance of the phthalate ion near 291.5 mμ , the phenyl phosphate kinetic runs were performed in 4×10^{-3} molar solutions buffered at pH 4 with sodium acetate-acetic acid buffer. Samples of the phosphate ester solutions were sealed in glass tubes and immersed in a constant temperature bath thermostated at 60.0° C. At specific times samples were withdrawn from the bath and frozen until analyzed. The reactions were followed by the production of both inorganic phosphate and phenol. After diluting a 10 ml. sample in a 100 ml. volumetric flask, samples were prepared for the phosphate and phenol determinations such that convenient readings could be obtained on the spectrophotometer.

Calculations

The reactivity ratio, R, of methyl alcohol to water could be calculated by the following equation.

$$R = \frac{\frac{\text{Conc. MeOH}}{\text{Conc. } H_2O} \times \text{Conc. phenol}}{\text{Conc. Phenol} - \text{Conc. phosphate}}$$

From the first-order kinetics equation

$$\frac{dx}{dt} = -kt$$

the following relationship can be derived

$$\ln \frac{X}{X_0} = -kt \quad . \qquad (1)$$

Equation 1 can be expressed in terms of products as

$$\ln \frac{(P_i - P_t)}{X_o} = -kt \; . \qquad (2)$$

Upon integrating Equation 2 and evaluating the intergration constant at $t = 0$, we obtain

$$\ln(P_i - P_t) = -kt + \ln P_i \qquad (3)$$

If a fraction of the products is captured then $P_i = (1-y)\, X_o$. Substituting in Equation 3 we have

$$\ln(P_i - P_t) = -kt + \ln (1-y)\, X_o \; . \qquad (4)$$

Clearly both Equation 3 and Equation 4 are of the form

$$y = mx + b.$$

Thus, when one product of the rate-determining step (metaphosphate ion) is trapped in the hydrolysis reaction a plot of the reaction following the production of both products will result in two parallel lines of equal slope. (The rate constants will be the same since the rate-determining step is the same.) The intercepts are different, a difference proportional to the ratio of the concentrations of the two products, inorganic phosphate and phenol.

Typical results of the kinetic runs for both the para-nitrophenyl phosphate and phenyl phosphate are tabulated in Tables 1 and 2.

Rate constants for the hydrolysis of para-nitrophenyl phosphate and phenyl phosphate in water and five per cent methyl alcohol solutions are shown in Table 3.

Table 1. Phenyl Phosphate Kinetic Run

Time Hr.	$[PO_4^{-3}] \times 10^6$	$[ROH] \times 10^6$	$[PO_4^{-3}]/[ROH]$	R
0	----	----	----	----
9	320	264	1.2	----
28	620	643	0.96	1.73
45	900	972	0.93	3.02
56	1080	1267	0.85	6.00
81	1324	1401	0.94	2.26
103	1552	1648	0.94	2.26
141	1836	2027	0.91	3.90
196	2248	2439	0.92	3.45
224	2470	2488	0.99	0.43
Infinity	3470	3780	0.94	2.26

Table 2. para-Nitrophenyl Phosphate Kinetic Run

Time Hr.	$[PO_4^{-3}] \times 10^6$	$[ROH] \times 10^6$	$[PO_4^{-3}]/[ROH]$	R
0	----	----	----	----
2.0	502	470	1.06	----
5.0	1062	1074	0.98	0.85
8.5	1522	1644	0.93	3.02
12.0	2052	2194	0.93	3.02
16.0	2452	2644	0.94	2.26
24.0	3472	3534	0.98	0.86
30.0	3752	3924	0.96	1.73
39.0	4152	4364	0.95	2.15
50.0	4502	4814	0.94	2.26
64.0	4822	5144	0.94	2.26
Infinity	5800	5520	0.95	2.15

Table 3. Reaction Rate-Constants in Mixed Solvents

Phosphate ester	k_{water} sec.$^{-1}$ water	k sec.$^{-1}$ Methyl alcohol
Phenyl	2.78×10^{-6}	1.97×10^{-6}
<u>para</u>-Nitrophenyl	1.28×10^{-5}	1.12×10^{-5}

A plot of log $(P_i - P_t)$ <u>vs</u>. time resulted in two parallel lines as predicted. These plots are shown in Figure 2 and Figure 3.

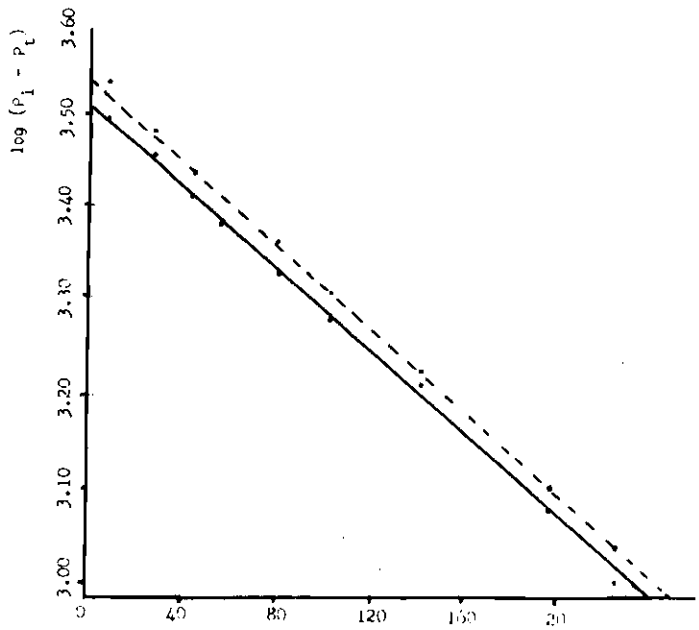


Figure 2. Plot log $(P_i - P_t)$ <u>vs</u>. Time, Phenyl Phosphate

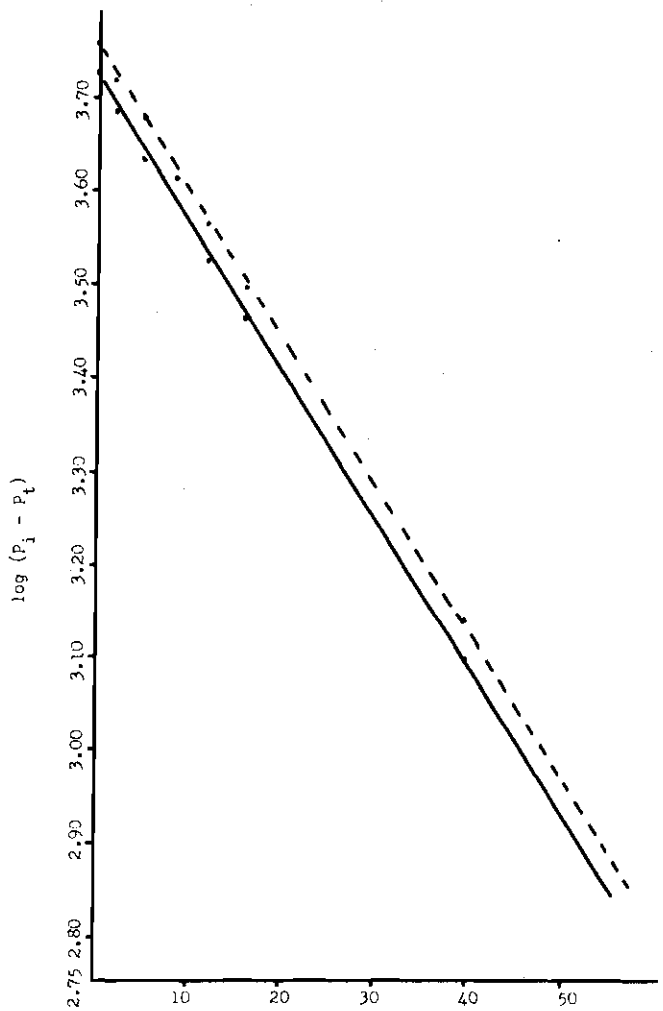


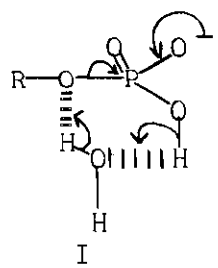
Figure 3. Plot log (P_i - P_t) vs. Time, para-Nitrophenyl Phosphate

CHAPTER III

CONCLUSIONS

As shown by the data in Tables 1 and 2 and by the graphs in Figures 2 and 3, the ratio of the alcohol formed to that of inorganic phosphate formed in five per cent methyl alcohol is essentially the same for both the hydrolysis of the phenyl phosphate and the para-nitrophenyl phosphate although the two esters are fairly different in absolute reactivity.

These results substantiate a mechanism in which an intermediate is formed in a rate-determining step which preceeds the product-determining step. An attractive form of this mechanism is that proposed by Kumamoto and Westheimer (Equation 1).



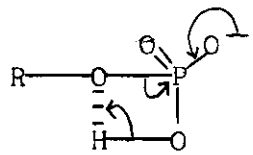
I

$$\longrightarrow \quad PO_3^- + ROH + H_2O \qquad (1)$$

The formation of the metaphosphate ion and the alcohol is the rate-determining step. The metaphosphate ion then reacts with either water or methyl alcohol in the product-determining step (Equation 2).

$$PO_3^- \xrightarrow[H_2O]{CH_3OH} \begin{matrix} CH_3OPO_3H^- \\ H_2PO_4^- \end{matrix} \qquad (2)$$

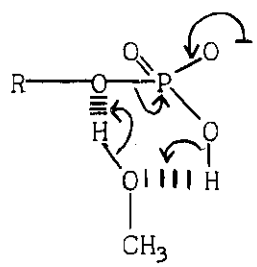
The evidence of the product ratios, however, does not distinguish between a mechanism in which the proton is transferred directly from the un-ionized acid group to the ester oxygen and one in which the proton transfer is effected via the cyclic intermediate (I). Either mechanism would be completely consistent with the product ratios observed as metaphosphate ion is also formed in the rate-determining step (Equation 3).



$$\longrightarrow \quad ROH + PO_3^- \qquad (3)$$

The metaphosphate can then react as shown in Equation 2 to form the same products.

The observation of a significant depression of rate by a small amount of methyl alcohol, an effect apparently not noted in earlier work, offers further evidence in support of the mechanism of Kumamoto and Westheimer (Equation 1). The observed decrease in rate could, for instance, be accounted for by disruption of the hydrogen-bonding of water to the monoanion by methyl alcohol. Or methyl alcohol could hydrogen bond to the monoanion as shown in Equation 4 to form an intermediate, II, which might be less reactive than that formed with water, but would still decompose to the same metaphosphate ion, although more slowly.



$$\longrightarrow \quad ROH + CH_3OH + PO_3^- \qquad (4)$$

II

The mechanism involving direct proton transfer from the unionized acid group (Equation 3) would not be expected to show any considerable effect on the rate of the reaction, as the solvent is not involved in the rate determining step. However, a mechanism which requires nucleophilic attack of the solvent on phosphorus would have yielded different product ratios from different esters as the rate-determining step is also the product-determining step.

Chanley and Feageson (1) recently found that the ratio of methyl phosphate to phenol formed was essentially the same for both para-nitrophenyl phosphate and phenyl phosphate in solutions varying from 30 to 70 per cent in methyl alcohol. The ratio of reactivity of methyl alcohol to water was reported as 1.18, 1.17, and 1.48 for solutions containing 70, 50, and 30 per cent methyl alcohol respectively. The ratio of reactivity of methyl alcohol to water as found in the present study was 2.5 for a system containing five per cent methyl alcohol.

It appears in the study by Chanley and Feageson that the reactivity of the methyl alcohol increases as the concentration of methyl alcohol decreases. The results described here are in accord with this observation. However, in the work by Chanley and Feageson the product ratios were determined only after the reaction had gone to completion and at a few intermediate points during the reaction. In the present study the reaction was followed over the entire course; hence any experimental errors would tend to be cancelled thus giving more reliable results.

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1. J. D. Chanley and Edward Feageson, J. Am. Chem. Soc., 85, 1181 (1963).

It is evident from the observed rate constants tabulated in Table 3 that even in five per cent methyl alcohol solutions the reaction rates of the systems under investigation are significantly changed from that of water. The discrepancy of the two studies, therefore, probably results from differences in the solvation of the intermediate, metaphosphate ion. That a more accurate reactivity ratio for water and methyl alcohol can be obtained from solutions in which the methyl alcohol concentrations are reduced to 10^{-2} molar seems apparent. The solvent properties of such a solution should not be different from that of water.

The methyl phosphate produced in the reaction can be measured directly and accurately using isotopic dilution techniques by employing C^{14} methyl alcohol in very small concentrations. Thus, with an accurate determination of methyl phosphate and the elimination of all solvent effects, the two disadvantages of the techniques used in the present study can be eliminated.

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*See Chemical Abstracts, 55, Pt. 9, (1961). "List of Journals" for standard abbreviations.